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Clusterin depletion enhances immune glomerular injury in the isolated perfused kidney

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Clusterin depletion enhances immune glomerular injury in the isolated perfused kidney. Clusterin is a normal plasma protein, shown to be an inhibitor of reactive complement hemolysis and a component of the fluid phase SC5b-9 terminal complement complexes. It is a component of glomerular immune deposits in human and experimental glomerulonephritis. Using the complement-dependent isolated perfused rat kidney model of autologous phase passive Heymann nephritis, we have studied the effect of clusterin depletion of perfused plasma on the development of glomerular injury. Kidneys with planted glomerular sheep anti-rat Fx1A antibody were perfused with human plasma either depleted of clusterin to ≤ 30%, or control plasma depleted of plasma fibronectin. Glomerular injury was then initiated by the addition of guinea pig anti-sheep immunoglobulins to the perfusate. Kidneys perfused with clusterin depleted plasma developed significantly greater proteinuria at all time points when compared to control kidneys. Glomerular antibody binding and C3 deposition were similar in the two groups, but terminal complement components were deposited in larger amounts in the clusterin depleted group. These data support a possible role for clusterin *in vivo* in the protection of complement-induced glomerular injury.

Human clusterin (SP-40,40) is a normal plasma protein which was first identified by the production of murine monoclonal antibodies (mAb) raised against the pathological glomerular basement membrane of a patient with membranous glomerulonephritis [1]. Clusterin was previously identified in the reproductive tracts of the rat and ram as a major secretory product of rat Sertoli cells in culture and a promoter of cell aggregation in ram rete testis fluid, hence the name clusterin [2].

Subsequently numerous groups have cloned homologues of the same protein in a variety of mammalian species and in a surprising variety of biological systems. The various names given to this protein are listed in Table 1.

In the kidney, clusterin has been found in two principal situations: in association with SC5b-9 terminal complement complexes and acutely in areas of renal injury [18, 19]. Clusterin has been detected both in glomerular immune deposits and also in extraglomerular sites of SC5b-9 deposition in the absence of immunoglobulin and early complement components. In one study of human renal biopsies glomerular clusterin deposition corresponded exactly to that of C5b-9 components and vitronectin [18]. Another recent biopsy study suggested that there was also an association between glomerular IgG and clusterin deposition [20]. In the experimental model of passive Heymann nephritis in the rat, clusterin deposition was seen in the glomeruli in association with C5b-9 and proteinuria. In this study decomplementation, which prevented C5b-9 deposition and proteinuria, was also associated with an absence of glomerular clusterin [21].

In vitro clusterin has been demonstrated to be an inhibitor of C5b-6 reactive hemolysis [22, 23]. It has been proposed that, analogous to vitronectin, clusterin binds to the unstable C5b-7 complex inhibiting the insertion of the complex into phospholipid cell membranes. Clusterin is then incorporated, together with vitronectin, into the soluble non-lytic SC5b-9 complement complex [22, 23].

It is possible, therefore, that clusterin *in vivo* is acting as a regulator of C5b-9 induced cell injury, and its presence in glomerular immune deposits represents a host limitation of complement injury by preventing C5b-9 insertion into cell membranes in the glomerulus.

We sought to test this hypothesis in a functional model of experimental glomerulonephritis where depletion of plasma clusterin would be achievable. The previously described [24], C5b-9 dependent, isolated perfused kidney model of autologous phase passive Heymann nephritis using perfused human plasma as a source of complement seemed well suited to this purpose. In this model, we have studied the effect on immune glomerular injury of depletion of clusterin from the perfused human plasma.

Methods

Kidney perfusions

The technique of kidney perfusion was as described previously by Cybulsky et al [24] with the exception that depleted and diluted human plasma was used to completely replace the standard BSA perfusate after establishment of perfusion. In the previous study [24] an equal volume of human plasma was added into the BSA perfusate after commencement of perfusion.

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Table 1. Synonyms of clusterin

Name	Reference
Clusterin	[3]
SGP-2 (sulphated glycoprotein 2)	[4]
	[5]
GP-III (glycoprotein III)	[6, 7]
TRPM-2 (testosterone-repressed prostrate message)	[8, 9]
Gp 80 (glycoprotein 80 kD)	[10]
SP-40,40 (serum protein having two 40 kD subunits)	[1]
CLI (cytolysis inhibitor)	[23]
T64 (message induced by *v-src* oncogene)	[11]
Apo J (apoliprotein J)	[12, 13]
pADHC-9, pTB-16, K-661 (gene clones)	[14–17]

BSA perfusate

The following preparation of the BSA perfusate was based on that used by Cybulsky [24].

A 20% BSA (Sigma) solution which had been extensively dialyzed against Krebs-Henseleit buffer (KHB) (5 times 5 vol for a total of 72 hr) was diluted in KHB to produce a final concentration of BSA of 80 mg/ml. Amino acids, glucose and calcium chloride were added to achieve a final composition in the BSA perfusate of (in mmol/liter); glucose 5.0; calcium. 3.0; sodium 140; magnesium 1.2; chloride 119; inorganic phosphate 1.2; SO_4 1.2; HCO_3 25, with the amino acids lysine 2.0; tyrosine 0.48; glutamine 4.2; glutamate 1.1; aspartate 0.4; asparagine 0.4; serine 2.0; cysteine 1.5; glycine 4.6; histidine 0.6; threonine 0.7; tryptophan 0.18; leucine 1.12; phenylalanine 0.72; isoleucine 1.0; methionine 0.6; valine 1.0; arginine 2.6; proline 1.2; and alanine 4.6.

Verapamil (Knoll AG. Germany) was also added to a final concentration of 10^{-4} M to prevent renal vasoconstriction [24].

Prior to use, perfusate was filtered through 8 μm and 0.45 μm filters (Millipore).

Preparation of human plasma

Two hundred fifty milliliters of fresh normal human plasma in acid citrate dextrose were collected by centrifugation of 500 ml of fresh blood (1000 g, 10 min) taken from a normal volunteer on the day prior to the perfusion. Sodium ethylenediaminetetraacetic acid (EDTA) was made 10 mM in the plasma and the plasma divided into two portions. Half of the plasma was depleted of clusterin and the other half depleted of fibronectin by affinity depletion chromatography as described below.

Depletion of plasma clusterin. Clusterin depletion was performed using a 10 ml column of cyanogen bromide activated Sepharose 4B (Pharmacia, Sweden) to which 80 mg of the G7 anti-clusterin monoclonal antibody [1] had been coupled according to the manufacturer's instructions. The column was equilibrated in 0.1 M phosphate buffered saline containing 10 mM EDTA (PBS-EDTA) and plasma passaged though in 10 ml aliquots. Each aliquot was washed through the column with PBS-EDTA and collected. The column was then eluted with 5 ml glycine HCl (pH 2.8) and re-equilibrated with PBS-EDTA before passage of another 10 ml of plasma. All procedures were carried out at 4°C. After all plasma had been passaged through the column, it was pooled and dialyzed overnight against 10 volumes of Krebs-Henseleit buffer (KHB) (without calcium) prior to use in the perfusion the following day. Dilution of the plasma during the affinity depletion resulted in a reduction in total plasma protein concentration to 55 to 60% of the initial level. Samples of the depleted plasma were stored at −20°C for later quantitation of the degree of clusterin depletion.

Control plasma depletion of plasma fibronectin. To control for complement inactivation by passage through affinity columns the control plasma was treated exactly as the clusterin depleted plasma with the exception that the affinity column was coupled to 80 mg of the monoclonal antibody against plasma fibronectin PHM 13 [25].

Quantitation of clusterin and fibronectin depletion. Following affinity depletion, samples of each plasma before and after affinity depletion were assayed by ELISA as previously described [1] to determine the proportion of plasma clusterin or fibronectin removed during each chromatographic procedure.

Modification of plasma prior to perfusion. After dialysis, clusterin depleted and control plasma were made up to a final albumin concentration of 60 mg/ml by the addition of a 20% BSA (Sigma) in KHB. In addition heparin (6 U/ml) (Commonwealth Serum Laboratories, Melbourne, Australia), verapamil 10^{-4} M (Knoll AG., Germany), and additional amino acids [(all in mmol/liter); lysine 1.0; tyrosine 0.24; glutamine 2.1; glutamate 0.5; aspartate 0.2; asparagine 0.2; serine 1.0; cysteine 0.75; glycine 2.3; histidine 0.3; threonine 0.35; tryptophan 0.09; leucine 0.6; phenylalanine 0.36; isoleucine 0.5; methionine 0.3; valine 0.5; arginine 1.3; proline 0.6; and alanine 2.3] were added together with glucose 5 mmol/liter and calcium 2.5 mmol/liter.

The plasma was filtered through 8 μm and 0.45 μm filters (Millipore) immediately before use.

Perfusion set up and animals

The perfusion circuit was as described by Ellis and Adam [26] and Cybulsky et al [24]. Experiments were carried out in a 37°C cabinet. Two peristaltic pumps were used to drive the perfusion circuit (Extech equipment, Masterflex), which included tubing (0.187 inch ID × 0.313 inch OD) connecting a glass perfusate reservoir, a glass film-oxygenator, an 8 μm Millipore filter, a flowmeter and a side arm pressure gauge. All glassware was siliconized and the perfusate was gassed with 95% O_2/5% CO_2 mix.

Three days prior to perfusion, male Sprague-Dawly rats, weighing 300 to 340 g were injected intravenously with 6 mg of γ2 sheep anti-rat Fx1A [24]. On the day of perfusions rats were anaesthetized with an intraperitoneal injection of pentobarbitone sodium (Boehringer Ingelheim, Australia; 0.12 ml/100 g). After an intravenous injection of 500 mg/kg mannitol, the right ureter was cannulated with a PE10 catheter. The renal artery was then cannulated with a glass cannula via the superior mesenteric artery without interrupting blood flow. Perfusion with BSA perfusate was commenced, the cannula tied in place and the kidney and ureter dissected out and mounted in the 37°C cabinet.

The kidney was initially perfused with the BSA perfusate for an equilibration period of approximately ten minutes. After this period the perfusate was changed to the appropriate plasma preparation. This was achieved by removal of the reservoir return tube and flushing of the circuit with plasma perfusate for a period of approximately five minutes. The kidney continued to be perfused during this time. After this time the return tube

was replaced and the reservoir filled with plasma. After 10 minutes perfusion with the plasma, 4 ml of heat-inactivated guinea pig anti-sheep IgG, as whole antiserum containing 2 mg/ml of IgG, was then added to the perfusate to initiate glomerular injury and measurements made.

During the two hour perfusion, perfusion pressure was adjusted to, and maintained at 95 to 99 mm Hg.

The perfusions were continued for a period of two hours, during which samples of perfusate and urine were collected every twenty minutes.

Adequacy of perfusions. Technically adequate perfusions were defined as having a glomerular filtration rate (GFR) greater than 0.4 ml/min, a urine flow rate (UFR) comparable between the two kidneys of each pair, a renal vascular resistance (RVR) of less than 5 mm Hg · min/ml and sodium reabsorption (FR_{Na}) greater than 75%.

Experimental design

All experiments were paired using separate perfusion circuits. In this way plasma from the same donor, collected and depleted the previous day, was used in experimental (clusterin depleted serum) and control (fibronectin depleted serum) perfusion at the same time.

Measurements

Glomerular filtration rate (GFR) was estimated by measurement of inulin clearance using [^{3}H] methoxy-inulin (Amersham, England, UK). Protein concentration in the urine samples were determined by the Lowry assay [27] and expressed as protein excretion rate (mg/min). Sodium in urine and perfusate samples was measured by a flame photometer. Perfusate flow rate was recorded throughout and renal vascular resistance (RVR) was calculated from the flow rate and perfusion pressure. Fractional sodium reabsorption (FR_{Na}%) was calculated from standard formulas. Urine flow rate (UFR) was calculated from the volume of urine excreted during each twenty-minute time interval (ml/min).

Production of antibodies

Monoclonal antibodies G7 and PHM13, G7 and PHM13, used to deplete plasma of clusterin and fibronectin, respectively, have previously been characterized [1, 25]. Both of the monoclonal antibodies are of the IgG1κ isotype and were prepared for coupling to the affinity matrix by ammonium sulphate precipitation of ascites fluid, dialysis against 0.1 M $NaHCO_3$, 0.5 M NaCl and adjusted to a protein concentration of 8 mg/ml with the same buffer.

γ2 Sheep anti-rat Fx1A. A sheep was immunized by repeated monthly injections of rat Fx1A, prepared as previously described [28]. After collection of sheep antiserum, the γ2 IgG non-complement fixing subclass was isolated by ion-exchange chromatography, heat-inactivated (56°C, 30 min) and concentrated as described [28]. All of the antibody was pooled, aliquoted and frozen at −70°C until use. In pilot experiments a maximal subnephritogenic dose was determined to be 6 mg per 300 g rat.

Guinea pig anti-sheep IgG, whole anti-serum. Hartly guinea pigs (Monash University Animal Services Center) were immunized by repeated four monthly subcutaneous injections with 1 mg purified sheep IgG (Silenus, Melbourne, Australia) in incomplete Freund's adjuvant (Sigma, Melbourne, Australia). Serum was collected three weeks after the last immunization, pooled, aliquoted and stored at −70°C until use. Prior to use, serum was heat-inactivated for one hour at 56°C.

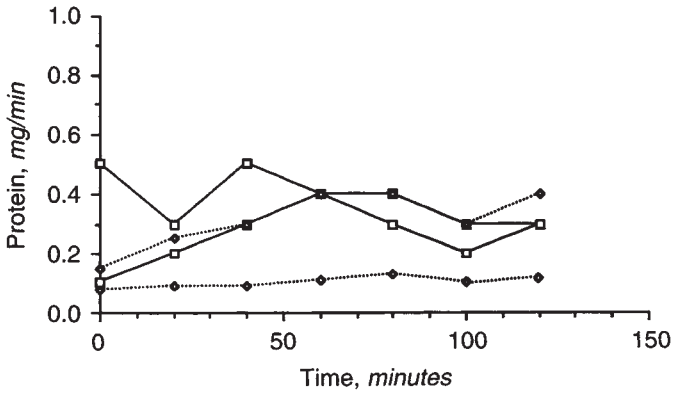

Fig. 1. *Protein excretion in kidneys perfused with complement inactive plasma.* Protein excretion was low and no significant difference was seen between clusterin (□; $N = 2$) or fibronectin (◇; $N = 2$) depleted plasma.

Affinity isolated guinea pig anti-sheep antibody was also produced for radiolabeling in glomerular binding experiments. Normal sheep IgG (Silenus) was coupled to cyanogen bromide Sepharose-4B (Pharmacia, Sweden) as per manufacturer's instructions (7 to 10 mg IgG coupled to 10 ml matrix). The whole guinea pig antiserum was passaged through the column and bound antibody eluted with 1.75 M KSCN [24].

Complement assays

Total hemolytic complement activity of human plasma samples pre- and post-depletion was measured using Quantiplate, Total Complement Test Kit (Kallestad Laboratories Inc., Austin, Texas, USA).

Concentrations of the third and fourth components of complement (C3 and C4) were measured in plasma samples using a Nephelometer (Behring) by the Clinical Immunology Laboratory, St. Vincent's Hospital.

Glomerular antibody binding

Glomerular binding of γ2 sheep anti-Fx1A to glomeruli. Sheep anti-Fx1A, γ2 fraction, 20 mg was iodinated with 4 mCi of I^{125} (Amersham) by the lacto-peroxidase technique [29]. This was diluted with 20 mg cold antibody and 6 mg per rat injected into six 300 to 340 g Sprague-Dawly rats. Specific activity was 0.1 μCi/μg (2.2×10^5 cpm/μg of protein).

Antibody binding was determined as described [30]. Three days after injection of labeled antibody the rats were sacrificed. Glomeruli were isolated by differential sieving [30], counted by phase contrast microscopy and I^{125} activity counted in a gamma counter (Packard, AutoGamma 5110). Glomerular antibody binding was expressed as micrograms per 38,000 glomeruli (the average number of glomeruli per rat kidney [31]).

To control for non-specific binding of serum proteins, an additional four rats were injected each with 6 mg ^{125}I labeled BSA (2.0×10^5 cpm/μg of protein). Three days later, glomeruli were isolated and counted as in the ^{125}I sheep anti-Fx1a experiments above.

Table 2. Percentage concentration of fibronectin and clusterin remaining in plasma after depletion

	Percentage remaining (after affinity depletion) of original plasma concentrations of clusterin/fibronectin	C3	C4	Total hemolytic complement *U/ml*
Normal plasma	—	0.83–1.77	0.11–0.45	> 35
Clusterin depleted plasma	25.9 ± 1.9%	0.26 ± 0.02	0.04 ± 0.003	26 ± 3
Fibronectin depleted plasma	23.0 ± 2.3%	0.27 ± 0.02	0.04 ± 0.002	26 ± 5

Comparison of percentage remaining after depletion of clusterin and fibronectin and comparison between the groups of concentrations C3c, C4(g/liter) and total hemolytic complement activity (hemolytic units) are from the eight group 2 perfusions.
No significant difference was seen between the two groups of depleted plasma.

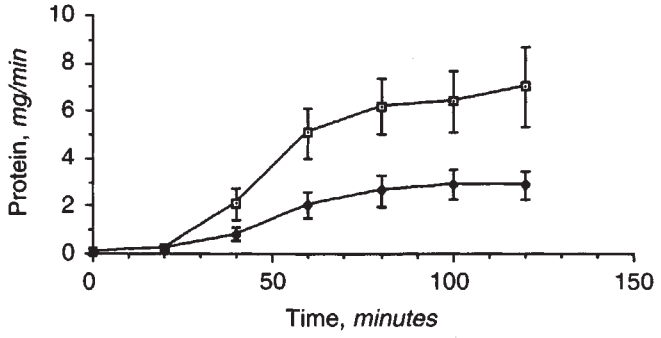

Fig. 2. *Eight pairs of kidneys perfused with fresh clusterin (□ ; N = 8) and fibronectin (● ; N = 8) depleted plasma.* Mean protein excretion rate with standard error over time of perfusion. Abnormal proteinuria develops in both groups of perfused kidneys and is a significantly greater when the kidneys are perfused with the plasma depleted of clusterin.

Glomerular binding of guinea pig anti-sheep IgG. Guinea pig anti-sheep IgG (5 mg) was affinity purified as described above and iodinated with 1 mCi I^{125} (Amersham) by the lacto-peroxidase technique [29]. Three pairs of perfusions were performed using the iodinated antibody, 2 mg of which was diluted into aliquots of 4 ml of cold guinea pig anti-sheep IgG antiserum. The specific activity was 0.1 μCi/μg (2.2×10^5 cpm/μg of of protein). Glomerular antibody binding was calculated and expressed as the number of glomeruli per rat kidney.

Histology, immunohistology, and electron microscopy

Immediately after completion of each perfusion, kidneys were sectioned and portions taken for conventional histology, immunohistochemistry and electron microscopy.

Immunofluorescence. For immunofluorescence, kidney tissue was immersed in OCT compound (Miles, USA) and snap frozen in dry ice-isopentane. Four micrometer frozen sections were cut and fixed in acetone for ten minutes. Direct immunofluorescence was performed with the following fluorescein isothiocyanate conjugated (FITC) antisera: donkey anti-sheep IgG, sheep anti-guinea pig IgG and sheep anti-human C3 (all from Silenus).

Immunohistochemistry (APAAP technique). Kidney tissue was fixed four hours in paraformaldehyde-lysine-periodate (PLP) and equilibrated for 72 hours in 7% sucrose buffer before being frozen in OCT compound (Miles) and dry ice-isopentane [32]. The alkaline phosphatase anti-alkaline phosphatase (APAAP) technique was used with monoclonal antibodies for the detection of human complement components C8 (produced in our laboratory), C9 [33], clusterin [1], vitronectin [22], C5b-9/C9 neoantigen (Quidel, San Diego, California, USA), and fibronectin [25].

Frozen sections of 4 μm, PLP fixed tissue were fixed in acetone for ten minutes. Incubation with mAb was followed by successive incubations with a bridging antibody, rabbit anti-mouse immuoglobulins (DAKO Z456) and the APAAP complex (DAKO D651). The reaction product was developed with substrate (Bio-Genics HK182-5K) and sections were counterstained with Harris hematoxylin and mounted with Permanent Aqueous Mounting Medium (Bio/Can Scientific).

Scoring of immunohistology. Immunohistology and immunofluorescence were scored semiquantitatively (0, 1+, 2+, 3+, 4+) by the same observer who was unaware of the experimental group of the sections. Group results were then expressed as a median score.

Light microscopy. For light microscopy, portions of kidney were fixed in formol sublimate solution, dehydrated in graded alcohols, cleared with chloroform and infiltrated with paraffin. Two micron serial sections were cut and stained with hematoxylin and eosin [34].

Electron microscopy. For electron microscopy, sections were fixed for three hours in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, post-fixed in 0.1 M phosphate buffer followed by 2% osmium tetroxide, and embedded in araldyte/epon. Sections (68 nm) were cut using the Ultra cut E (Reichert-Jung) and stained with 0.23 M uranyl acetate in 50/50 methanol/ddH_2) and Reynolds lead citrate. A Philips CM 10 electron microscope was then used to examine the sections.

Statistics

Repeated measures analysis of variance was performed using the SuperAnova package (Abbacus Concepts Berkely, California, USA) statistical package to determine the significance of differences in protein excretion, GFR, RVR, and Na reabsorption between the two experimental groups. Repeated measures analysis of covariance, using the SysStat package version 5.2, was also performed in the clusterin depleted group to determine whether the degree of proteinuria was related to the degree of clusterin depletion.

Results

Three series of technically successful perfusions were performed.

The results of the perfusions are described in three separate groups: (1.) complement inactivated plasma; (2.) complement

Table 3. Isolated perfused kidney function

Period Time	1 0–20	2 20–40	3 40–60	4 60–80	5 80–100	6 100–120
Clusterin-depleted plasma group						
RVR	3.2 ± 0.2	3.8 ± 0.3	4.2 ± 0.3	4.6 ± 0.4	4.8 ± 0.4	5 ± 0.4
GFR	0.6 ± 0.1	0.68 ± 0.1	0.54 ± 0.1	0.56 ± 0.1	0.6 ± 0.1	0.5 ± 0.1
FR_{Na}	88 ± 1.2	83 ± 2.9	66 ± 6.4	66 ± 3.8	65 ± 4.1	59 ± 4.7
UFR	0.11 ± 0.02	0.16 ± 0.02	0.2 ± 0.02	0.23 ± 0.01	0.23 ± 0.02	0.23 ± 0.01
Fibronectin-depleted plasma group						
RVR	3.2 ± 0.2	3.3 ± 0.2	3.5 ± 0.2	3.7 ± 0.2	4 ± 0.2	4.1 ± 0.2
GFR	0.89 ± 0.1	0.66 ± 0.1	0.7 ± 0.1	0.56 ± 0.1	0.54 ± 0.1	0.55 ± 0.1
FR_{Na}	87 ± 2.3	83 ± 3	80 ± 3.5	74 ± 2.1	73 ± 2.9	70 ± 6.3
UFR	0.15 ± 0.02	0.15 ± 0.02	0.19 ± 0.04	0.2 ± 0.04	0.19 ± 0.03	0.17 ± 0.02

All values are expressed as mean ± SE. Abbreviations are: RVR, renal vascular resistance (mm Hg · min/ml); GFR, glomerular filtration rate (ml/min); FR_{Na}, sodium reabsorption (%); UFR, urine flow rate (ml/min). The function of the eight paired isolated kidney perfusions (group 2) are reflected in the GFR, RVR, FR_{Na} and UFR. For each of these variables a repeated measures analysis of variance was performed to determine whether there was any effect of group (clusterin depleted or fibronectin depleted). There was no significant effect (at the 0.05 level) of group on any of the functional parameters.

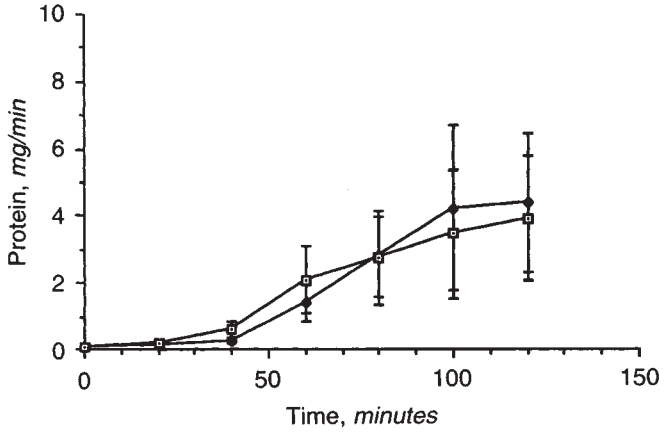

Fig. 3. *Three pairs of fresh plasma perfusions with "inadequate" clusterin depletion.* Mean protein excretion with standard error over the time of perfusion. There is no significant difference in proteinuria between kidneys perfused with fibronectin depleted plasma (●; $N = 3$) and those perfused with plasma inadequately depleted of clusterin (⊡; $N = 3$).

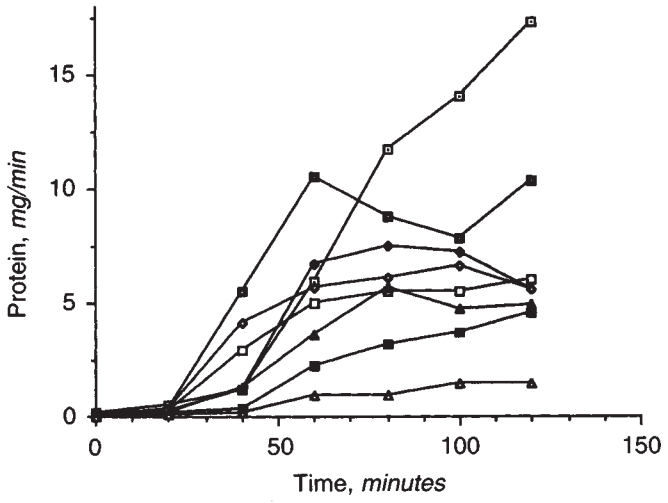

Fig. 4. *Relationship between degree clusterin depletion and proteinuria.* Symbols are: (⊡) 16%; (◘) 19%; (□) 26%; (◇) 28%; (▲) 30%; (◆) 30%; (■) 30%; (△) 28%. Individual plasma samples from perfusions were tested for clusterin depletion by ELISA and these data plotted against proteinuria over time for the individual perfusions.

active and adequately depleted plasma; and (3.) complement active plasma with inadequate clusterin depletion.

Adequacy of clusterin and fibronectin depletion and complement inactivation of some plasma samples

Depletion of plasma clusterin or fibronectin to 30% of the initial concentration was readily achievable with the affinity chromatography, and this level of depletion was arbitrarily selected to define adequate depletion for the experimental and control groups. Determination of the degree of depletion was performed after each perfusion; hence a series of three perfusions were performed with inadequate clusterin depletion due to a failure of the affinity column.

Determination of the complement activity of depleted plasma was also performed after completion of the perfusion, and two perfusions were performed with plasma which was subsequently found to be complement inactivated. The results of these perfusions do, however, serve as additional controls and are described as groups 1 and 3 below.

Group 1. Perfusions with complement inactivated plasma. It was initially intended to perform perfusions with heat-inactivated plasma (56°C, 45 min) to confirm complement dependance of this model. Heat-inactivation, however, resulted in protein aggregation which rendered the plasma non-filterable. A serendipitous complement inactive control group was identified in the series of two initial pairs of perfusions which were performed using plasma which had been passaged through the affinity columns without prior addition of EDTA. This resulted in significant complement activation on the affinity column and reduction in subsequent total hemolytic complement activity in both clusterin and fibronectin depleted plasma (<5%). On perfusion of kidneys with these plasma preparations, no increase in proteinuria occurred, as shown in Figure 1. The excreted protein levels remained low throughout the perfusion and there was no significant difference between clusterin and fibronectin depleted plasma.

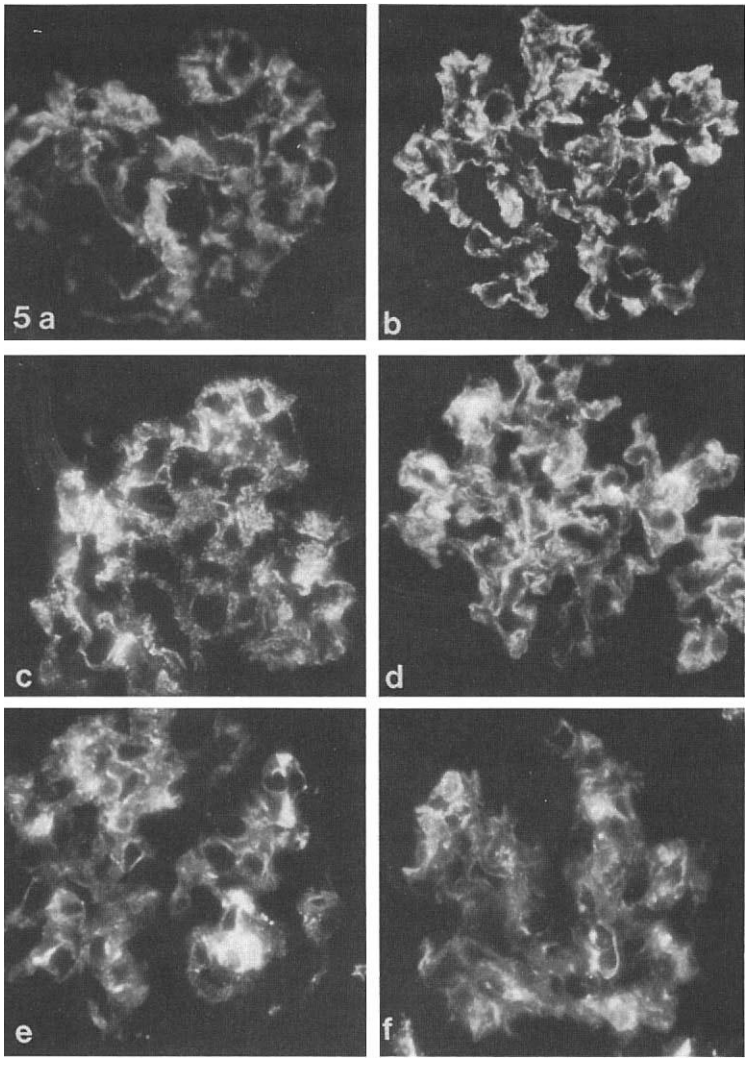

Fig. 5. *Direct immunofluorescence on sections of perfused kidneys.* **(a), (c)** and **(e).** Kidneys perfused with clusterin depleted plasma with detection of sheep IgG, guinea pig IgG and human C3c, respectively. **(b), (d)** and **(f).** Kidneys perfused with fibronectin depleted plasma with detection of sheep IgG, guinea pig IgG and human C3c, respectively. There are no differences between the groups.

Group 2. Clusterin-depleted complement active perfusions. Eight successful pairs of perfusions were performed using fresh human plasma that had been depleted the previous day, with all clusterin and fibronectin depletion to less than 30% of initial concentrations (range 30 to 15%). The total hemolytic complement activity, C3 and C4 levels for both groups are shown in Table 2. In both groups there was a significant increase in urinary protein excretion over the time of the perfusion. The kidneys perfused with clusterin depleted plasma, however, showed a significantly greater increase in proteinuria as compared to the fibronectin depleted control (Fig. 2). Analysis of variance showed a significant difference ($P < 0.01$) in the protein excretion between the groups. There was no significant difference, using repeated measures analysis of variance, between the groups with respect to GFR, RVR, Na reabsorption and UFR (Table 3).

Group 3. Inadequate clusterin depleted-complement active perfusions. Three successive paired perfusions were, retrospectively, found to have insufficient depletion of clusterin (80 to 70% of initial concentration) due to a failure of the G7 affinity column. Fibronectin depletion was adequate in these perfusions (depletion to ≤30%) and there was no difference in the total hemolytic complement activity, C3 and C4 levels between the groups. Proteinuria in these pairs, showed no significant difference between the clusterin and fibronectin depleted plasma perfusions (Fig. 3). The protein excretion in all perfusions in group 3 was comparable to that seen in the fibronectin-depleted plasma perfusions in group 2. These data suggest that "adequate clusterin depletion" was responsible for the enhanced proteinuria in group 2.

Correlation between the degree of clusterin depletion and proteinuria

To further analyze the effect of the degree of clusterin depletion on the resultant proteinuria, the data from group 2 were analyzed in relation to the degree of clusterin depletion. As can be seen in Figure 4, there was a relationship between the protein excretion rate in kidneys perfused with clusterin depleted plasma and the extent of the clusterin depletion. Using repeated measures analysis of covariance on the data in Figure 4, the percentage clusterin depletion was shown to have a significant independant effect on proteinuria ($P < 0.01$).

Table 4. Fluorescence and APAAP labeling of tissue sections of perfused kidneys and glomerular binding of guinea pig anti-sheep IgG

	Clusterin-depleted plasma perfused kidneys	Fibronectin-depleted plasma perfused kidneys	Kidneys perfused with complement inactivated plasma
Fluorescence			
ShIgG	++++	++++	++++
GPIgG	++++	++++	++++
Human C3c	+++	+++	trace
APAAP			
C8	++	+	−
C9	+++	+	−
C5b-9 neo.	+++	+	−
clusterin	−	−	−
vitronectin	trace	trace	trace
fibronectin	+	+	+
Glomerular binding of guinea pig anti-sheep IgG μg/38,000 glomeruli, mean ± SEM	3.1 ± 0.9	2.8 ± 1.1	not done

Histology, immunohistology and electron microscopy

Histological examination of kidneys from both the clusterin depleted and fibronectin depleted groups showed no significant abnormalities by light microscopy.

Immunofluorescence. All kidneys showed a granular deposition of sheep IgG (Fig. 5 a, b) and guinea pig IgG (Fig. 5 c, d). Human C3 (Fig. 5 e, f) was detected along the glomerular capillary walls in the kidneys from group 2, but only trace amounts were found in group 1 kidneys perfused with complement inactive plasma. In group 2, there was no detectable difference in fluorescence intensity between kidneys perfused with clusterin depleted plasma and those perfused with fibronectin depleted plasma (Fig. 5, Table 4).

Immunohistochemistry (APAAP). Deposition of the terminal complement components C8 and C9, along with the C5b-9 neoantigen (Fig. 6 a, b, c) were seen to be greater in the glomeruli of kidneys perfused with plasma depleted of clusterin (Table 4).

Clusterin deposition was seen in the vessels in both groups of kidneys, but there was no detectable glomerular clusterin in either group. Fibronectin was detected at low levels in the mesangium of glomeruli with no difference between kidney groups and vitronectin showed trace deposition (0.5+) in the glomeruli with no significant difference between the groups (Table 4).

Electron microscopy. Electron microscopy showed a difference between the two groups of perfused kidneys. Kidneys perfused with clusterin depleted plasma showed greater effacement and detachment of the podocyte foot processes compared to the kidneys perfused with the fibronectin depleted plasma (Fig. 7 A, B). Kidneys from both groups showed small subepithelial electron dense deposits (Fig. 7 arrows).

Glomerular antibody binding

Glomerular binding of γ2 sheep anti-Fx1A. Mean glomeruli antibody binding of the γ2 sheep anti-Fx1A in the five rats studied was 17 ± 2.3 μg/38,000 glomeruli. In the four control rats injected with ^{125}I BSA there was no detectable binding of BSA (<1 μg/38,000 glomeruli).

Glomerular binding of guinea pig anti-sheep IgG. Glomerular binding of guinea pig anti-sheep IgG, following perfusion with clusterin depleted plasma was 3.1 ± 0.9 μg/38,000 glomeruli, compared to a binding of 2.8 ± 1.1 μg/38,000 following perfusion with fibronectin depleted plasma. This difference was not significant.

Discussion

The initial perfusions in this study using complement inactive plasma confirmed previous findings that this model of PHN in the IPK is complement dependant [24].

In the main experiments (group 2) there was significantly greater proteinuria when perfusions were with plasma depleted of plasma clusterin to less than 30% of initial concentration. These data, together with the fact that the degree of clusterin depletion was related to the level of proteinuria, suggests that clusterin depletion enhanced glomerular injury in this model.

The binding studies and immunohistology showed similar binding of sheep and guinea pig IgG and of C3 in both clusterin and fibronectin depleted plasma perfused kidneys, despite the differences in protein excretion. Accordingly, it is unlikely that clusterin depletion affected the initiation of immune glomerular injury or the activation of early complement pathways. However, deposition of glomerular C5b-9/C9 neoantigen along with the individual terminal components C8 and C9 was significantly different between the two groups. Kidneys perfused with plasma depleted of clusterin showed greater deposition of C5b-9 components. This supports the hypothesis that the increase in proteinuria seen following depletion of clusterin is due to greater damage to the glomerular epithelial cells by C5b-9.

No glomerular clusterin deposition and only trace amounts of vitronectin were seen in any of the perfused kidneys. Clusterin and vitronectin are detected at day 5 in *in vivo* models of PHN [21], but it is unclear why the soluble SC5b-9 complex is an apparently stable component of glomeruli in the later stages of experimental glomerulonephritis and in human glomerulonephritis [18]. The absence of clusterin or significant amounts of vitronectin in the glomeruli of either of our IPK groups, despite significant C5b-9 deposition, suggests that all the C5b-9 is in the

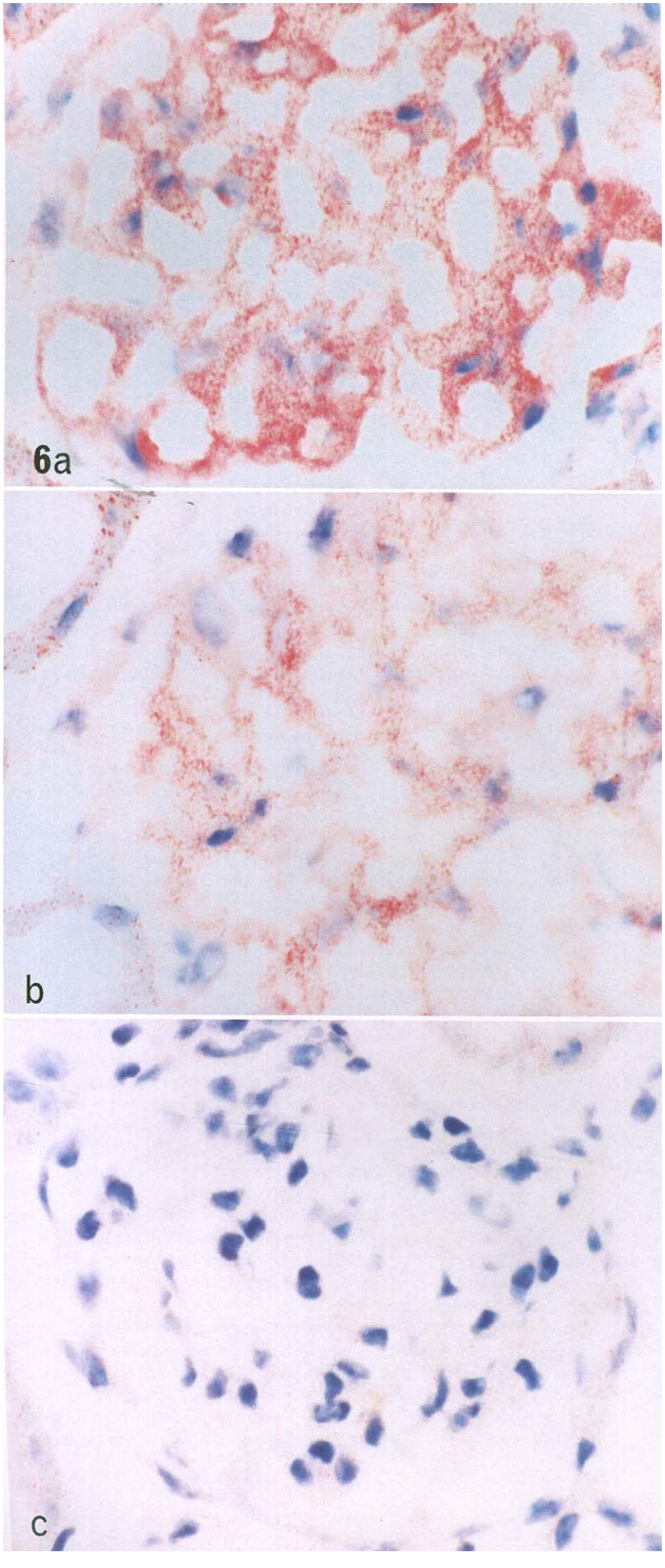

Fig. 6. *APAAP on PLP fixed perfused kidney sections*. Neoantigen C5b-9/C9 was deposited in greater amounts in the glomeruli of kidneys perfused with clusterin depleted plasma (**a**) when compared with fibronectin-depleted perfused kidneys (**b**). Kidneys perfused with complement inactive plasma contained no glomerular C5b-9 (**c**). Reproduction of this figure in color was made possible by a grant from Janssen-Cilag Pty. Ltd., Lane Cove NSW, Australia.

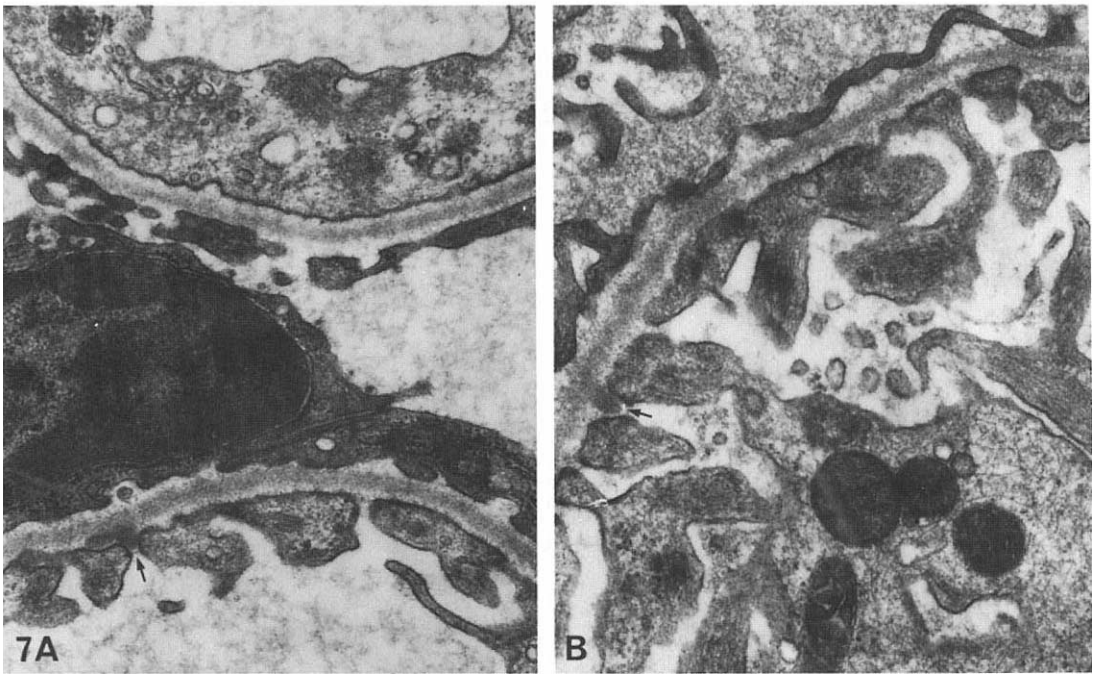

Fig. 7. *Electron microscopy shows marked effacement of epithelial cell foot processes in the clusterin-depleted plasma perfused kidneys (A), with lesser abnormality in the fibronectin-depleted plasma perfused kidneys (B).* Subepithelial deposits are seen in both groups of kidneys (arrows).

phospholipid membrane bound C5b-9(m) form. The failure to detect SC5b-9 complexes in the glomeruli could indicate that SC5b-9 complexes accumulate at a slow rate in this system and were therefore not detectable after only a two hour perfusion. Alternatively SC5b-9 complexes may have formed but did not remain in the glomeruli after perfusion and/or tissue processing.

Despite the absence of glomerular clusterin/SC5b-9 in either group, clusterin depletion of the perfused plasma was associated with significantly greater glomerular C5b-9(m) deposition. This suggests that clusterin may normally have an inhibitory effect on the assembly of C5b-9(m) on cell membranes or that some of the complement activation in this model occurs adjacent to the cell membrane, leading to "bystander" lysis. Bystander lysis is typically inhibited by fluid phase regulators of C5b-9 such as clusterin.

The ultrastructural studies confirm that greater abnormality of the podocytes was seen in the clusterin depleted plasma perfused kidney. These data accord with those of Cybulsky et al [24] who showed a relationship between proteinuria and podocyte damage.

It is possible that depletion of plasma fibronectin in our control group may have, in some way, decreased glomerular injury. This seems unlikely to be responsible for the differences observed as the proteinuria seen in the control (fibronectin depleted) group was similar to that seen when plasma was passaged through a column of uncoupled sepharose CnBr 4B (4 mg/min at 120 min) and was similar to the proteinuria observed by Cybulsky et al [24] in perfusions with 55% diluted human plasma. Furthermore the similarity in proteinuria between groups when clusterin depletion was "inadequate" (group 3) suggests that it was the clusterin depletion itself that was principally responsible for the difference in proteinuria.

There are a number of inhibitors of the C5b-9 that could potentially limit glomerular injury in this system. For example, rat podocytes presumably express the rat homolog of the GPI-linked membrane complement inhibitor CD59 [35, 36]. This molecule, at least in the human, functions principally as an inhibitor of lysis by homologous complement along with the other membrane inhibitors of the earlier complement pathways, for example decay accelerating factor [37, 38]. In this IPK system, with heterologous plasma perfusion, it is probable that the cell membrane related inhibitory proteins in the rat kidney would be less efficient inhibitors of complement activation. Indeed, it is very likely that, even in the absence of antibody administration, perfusion of rat kidneys with human plasma would result in significant complement activation as a result of naturally occurring xenoantibody and loss of homologous restriction. In this context, it is possible that the human soluble plasma inhibitors of the assembly of C5b-9, clusterin and vitronectin, may play a relatively more significant role in regulation of complement activation. As a result, a 30% depletion of clusterin is able to produce a substantial enhancement of complement mediated injury.

Whether or not the significance of clusterin as a C5b-9 inhibitor is artificially enhanced in this model, it is now clear from these data that clusterin is able to inhibit complement *in vivo*. This is the first demonstration of a likely functional complement inhibition by clusterin outside of an *in vitro* lysis system.

It is clear from recent work from many groups, that clusterin has numerous likely biological functions outside of the complement system [39, 40]. It binds to lipid and some lipoproteins, it is present in secretory vesicles of the adrenal medulla and canine kidney epithelial cells, it is constitutively expressed at high levels in the reproductive tracts and probably the brains of all mammals and is present in most human bodily secretions.

Taken together with the known increase in clusterin expression following cell injury, the suggestion has been made [41] that clusterin functions as a form of "membrane policeman" involved not only in the protection of membranes from complement, but also involved in membrane associated secretion, removal or remodeling of membranes during injury or development. It may therefore be that in these studies, the apparent partial protection from injury afforded by normal plasma clusterin concentrations was not wholly specific for complement-mediated cell injury. The role of clusterin in the control of non-complement-dependant glomerular cell injury is worthy of study in the future.

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